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TITLE: **CaMKK2 Inhibition in Enhancing Bone Fracture Healing**

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13. SUPPLEMENTARY NOTES ACURO approval was received on 12 August 2015, upon which the studies commenced.					
14. ABSTRACT Ca ²⁺ /calmodulin (CaM)-dependent protein kinase kinase 2 (CaMKK2) has roles in the anabolic and catabolic pathways of bone remodeling. We hypothesized that targeting CaMKK2 will result in accelerated fracture healing. We generated unilateral mid-shaft fractures using a three-point bending method (first described for use in rats by Bonnarens and Einhorn, 1986) in the right femurs of 10-week old anesthetized male mice after first inserting an intramedullary pin (25 gauge needle, approx. 0.5 mm) retrograde through the distal condyle of the femur. Radiographic analyses were performed to confirm the location and quality of the fractures. Since CaMKK2 inhibition is associated with anti-inflammatory phenotype, we wanted to determine the optimal time for STO-609 administration. Thus, fractured animals were divided into three groups: (a) saline only (n=15), (b) STO-609 from day 0 (n=15) and (c) STO-609 from day 7 (n=15). Tri-weekly intraperitoneal (i.p.) injections of saline or STO-609 (10 µmol/kg mouse body weight) were performed for 6 weeks. Progression of fracture healing was monitored through weekly radiographic examination. Fractured callus and non-fractured contralateral femur diaphysis were analyzed by micro computed tomography, histology, immunohistochemistry and histomorphometry. Preliminary results indicate that treatment with STO-609 results in the formation of a robust callus at the fracture site, indicating accelerated healing of femoral fractures following the pharmacological inhibition of CaMKK2.					
15. SUBJECT TERMS Femur fracture, CaMKK2, STO-609					
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1. Introduction: Fracture healing is a chief medical concern for active-duty military personnel as well as aging combat veterans. Fractures associated with osteoporosis and acute trauma result in significant medical costs, loss of productivity and most importantly, loss of patient quality of life. Prolonged healing time and non-union occur in 5-10% of these fractures, contributing to further medical costs and patient morbidity. Established therapies that treat osteoporosis such as bisphosphonates only reduce the risk of fractures by an average of 20% and they do not promote efficient bone growth and fracture healing. Consequently, there are no current pharmacological treatment options available to promote efficient bone fracture healing. Ca^{2+} /calmodulin (CaM)-dependent protein kinase kinase 2 (CaMKK2) has roles in the anabolic and catabolic pathways of bone remodeling. Pharmacological inhibition of CaMKK2 in female mice using its selective cell-permeable inhibitor STO-609 protects from ovariectomy-induced osteoporosis. Moreover, treatment of 32 week old male mice with STO-609 reverses age-associated decline in bone volume and strength. Based on these studies, we hypothesized that targeting CaMKK2 will result in accelerated fracture healing

2. Keywords: Femoral fracture healing, CaMKK2, STO-609

3. Accomplishments:

A. What are the major goals of the project:

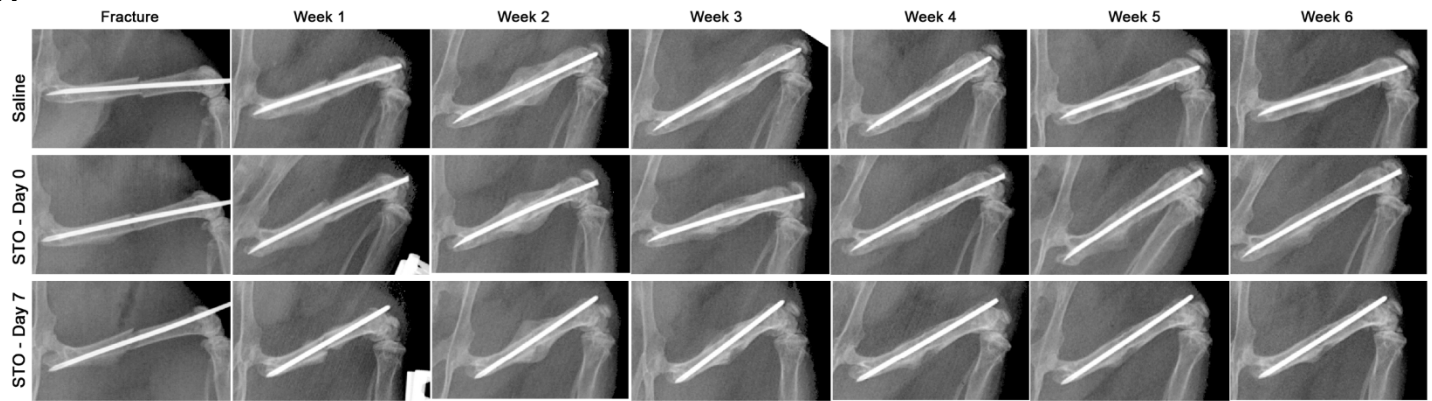
1. Specific Aim 1: Determine the optimal conditions of CaMKK2 inhibition through STO-609 that will confer the most efficacious recovery from bone fracture
2. Specific Aim 2: Effect of genetic ablation or pharmacological inhibition of CaMKK2 on post-fracture bone physiology and recovery

B. What was accomplished:

Eight week old male C57BL6 (50) were purchased from Harlan Laboratories (Indianapolis) and housed under a 12-hr light and dark cycle, with food and water provided *ad libitum*. All care and experimental procedures were performed according to Institutional Animal Care and Use Committee (IACUC) protocols and in compliance with NIH guidelines on the use and care of laboratory and experimental animals. At 10 weeks of age, the mice were anesthetized and an intramedullary pin (25 gauge needle, approx. 0.5 mm) was inserted retrograde through the distal condyle of the femur. Following this, unilateral mid-shaft fractures using the three-point bending method (as described in rats by Bonnarens and Einhorn, 1986) were generated. Radiographic analyses were performed to confirm the location and quality of the fractures. Fractured animals were divided into three groups: (a) saline only (n=15), (b) STO-609 from day 0 (n=15) and (c) STO-609 from day 7 (n=15). Tri-weekly intraperitoneal (i.p.) injections of saline or STO-609 (10 $\mu\text{mol/kg}$ mouse body weight) were administered and these treatments commenced either (a) from the day after the fracture (saline and STO-day 0) or (b) from the 7th day following fracture (STO-day 7). Treatments continued for 6 weeks post-fracture. Progression of fracture healing was monitored through weekly radiographic examination using Faxitron MX-20 with digital camera (Faxitron X-ray Corporation). Fractured callus and non-fractured contralateral femur diaphysis were analyzed by micro computed tomography at voxel size of 10 μm using Scanco μCT 35, followed by histology.

Results:

A



B

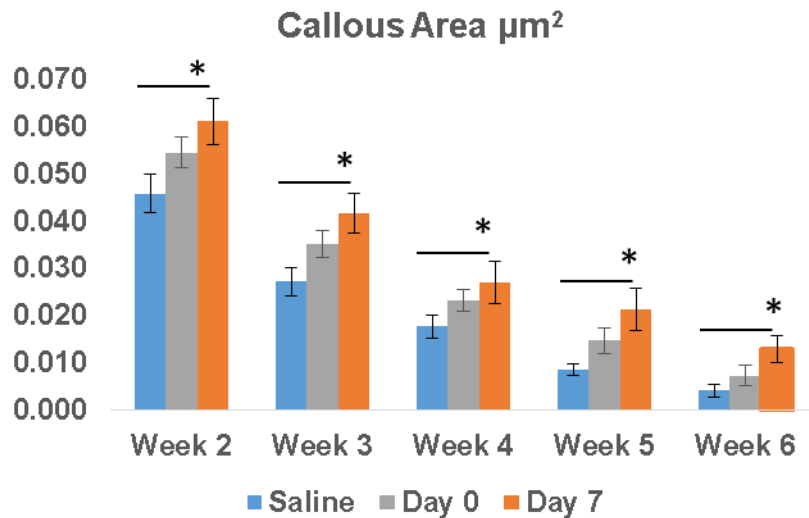
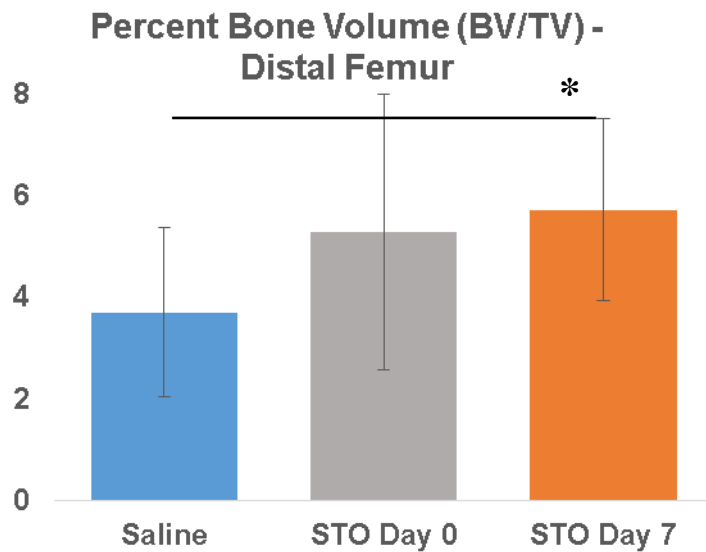


Figure 1: Effect of CaMKK2 inhibition on radiographical outcomes of fracture healing. (A)

Representative radiographical images of fractured femurs from mice treated with saline or STO-609 (day 0) and/or (day 7) taken weekly for 6 weeks from the same mice. (B) Average fracture callus area from saline and/or STO-609 treated mice over the 6 week treatment period. Radiograph images were (from A) were imported into ImageJ software (National Institutes of Health, Bethesda, MD) and any callus visible beyond the bone's original periosteal surface was traced. Image contrast was altered to facilitate delineation of the original periosteal surface and calluses. Statistical analysis performed using one-way ANOVA ($p < 0.05$). Error bars represent SEM; ($p < 0.05$).

A



B

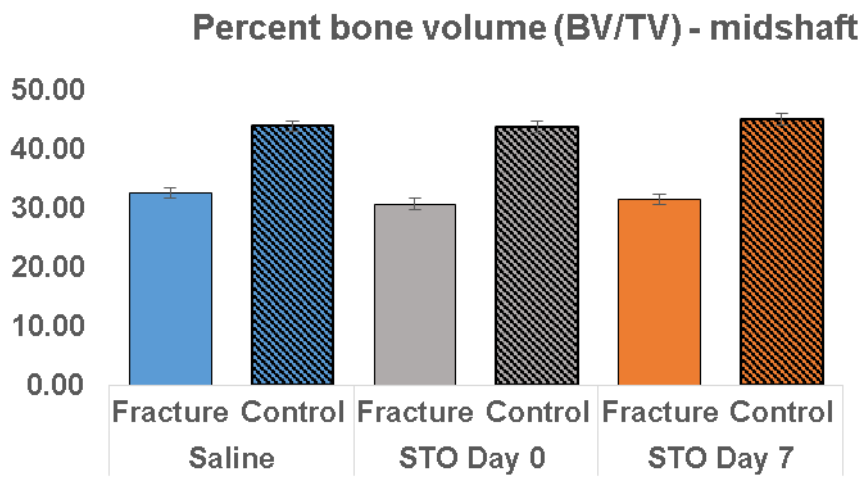
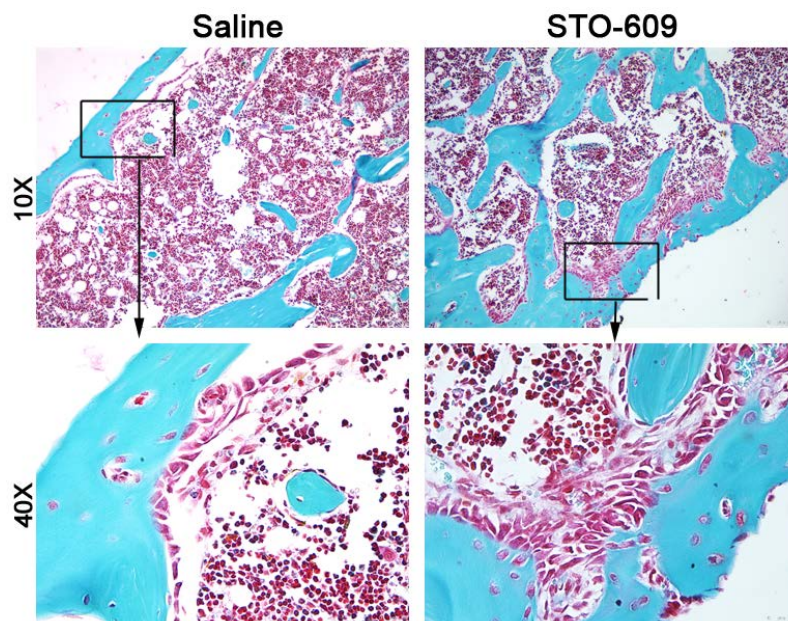


Figure 2: Effect of CaMKK2 inhibition on bone volume in fractured and contralateral mouse femurs. Mean percent bone volume calculated using micro computed tomography among mice treated with saline or STO-609 (day 0) and/or (day 7) for 6 weeks following fracture. (A) Trabecular bone at the distal end of contralateral femurs used to confirm effectiveness of STO-609. Average of $n=6$ per group. (B) Percent bone volume of fracture callus and non-fractured contralateral diaphysis. Bones were scanned ex-vivo after 6 weeks of treatment ($n=10$ /group). Statistical analysis performed using one-way ANOVA ($p<0.05$). Error bars represent SEM.



CaMKK2 inhibition enhances osteoblasts in fracture callus.

Femur sections at week 6 post fracture were stained with Safranin O/Fast Green and visualized. Representative of n=6 per group from saline or STO (day 7) shown.

Conclusions: In this study we investigated the effect of CaMKK2 inhibition on fracture healing. Our results thus far indicate that delaying treatment with STO-609 by a week post-fracture results in a significant increase in callus area (**Figure 1A-B**). They also indicate that CaMKK2 inhibition significantly enhances trabecular bone volume (**Figure 2A**) as well as trabecular thickness and number (data not shown) in contralateral distal femurs. Moreover, treatment with STO-609 results in the stimulation of osteoblasts lining the bone surfaces in the callus area (**Figure 3**). At the same time, we did not observe any difference in the callus bone volume among the different treatments at 6 weeks post-fracture (**Figure 2B**). One possibility is that the differences in callus volume diminishes over time and may not be detectable when fractures are assessed at 6 weeks.

C. What opportunities for training and professional development has the project provided:

Nothing to report.

D. How were the results disseminated to communities of interest?

Williams J, Cheng Y-H, Hassert M, Liu J, Li Y, Robling A, Kacena M and Sankar U. CaMKK2 inhibition enhances bone fracture healing. 37th Annual Meeting of the American Society for Bone and Mineral Research, October 9-12, 2015, Seattle, WA.

E. Plan for the next period:

Presently, we are analyzing earlier time points i.e., 2 and 4 weeks following fracture to assess whether STO-609 treatment accelerates bone healing. We are also performing a dose-response curve for STO-609 in fracture healing.

4. Impact

Nothing significant to report for this project period.

5. Changes/Problems

Nothing to report.

6. Products

Nothing to report.

7. Participants and other collaborative institutions

PI: Uma Sankar, Ph.D.

Associate Professor, Department of Anatomy and Cell Biology, Indiana University, Indianapolis, IN 46202.

Nearest person months worked: 4

Contribution to the project: Designed the study and interpreted the results. Wrote the report.

Collaborator: Alexander Robling, Ph.D.

Professor, Department of Anatomy and Cell Biology, Indiana University, Indianapolis, IN 46202.

Nearest person months worked: 1

Contribution to the project: Micro-CT analysis and data interpretation.

Collaborator: Melissa Kacena, Ph.D.

Professor, Department of Orthopaedics, Indiana University, Indianapolis, IN 46202.

Nearest person months worked: 1

Contribution to the project: Surgery, data collection and analysis.

Research Technician: Justin Williams, BS.

Department of Anatomy and Cell Biology, Indiana University, Indianapolis, IN 46202.

Nearest person months worked: 7

Contribution to the project: Performed experiments and collected data.

Research Scientist: Ying-hua Cheng, Ph.D.

Department of Orthopaedics, Indiana University, Indianapolis, IN 46202.

Nearest person months worked: 1

Contribution to the project: Performed fracture surgeries and injections.

Research Technician: Mariah Hassert, BS.

Department of Anatomy and Cell Biology, Indiana University, Indianapolis, IN 46202.

Nearest person months worked: 1

Contribution to the project: Performed experiments and collected data

Research Technician: Yong Li, MBBS.

Department of Anatomy and Cell Biology, Indiana University, Indianapolis, IN 46202.

Nearest person months worked: 1

Contribution to the project: Performed surgeries and injections

Research Technician: Jianying Liu, MBBS.

Department of Anatomy and Cell Biology, Indiana University, Indianapolis, IN 46202.

Nearest person months worked: 1

Contribution to the project: Performed surgeries and injections

A. Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Yes.

1. RSG13-301-01-TBG Research Scholar Grant
07/1/13-06/30/17 3.0 calendar

American Cancer Society

Role: PI

Title: CaMKK2 Inhibition in Palliative Care of Advanced Prostate Cancer Patients.

Aims: Development of CaMKK2 inhibition as a viable strategy to inhibit bone loss while suppressing tumor growth in patients with advanced stage prostate cancer on androgen deprivation therapy.

2. 1R01AR068332-01A1 NIH R01
09/18/2015 – 06/30/2020 3.6 calendar

NIH/NIAMS

Role: PI

Title: CaMKK2 Inhibition as a Dual-Action Bone Anabolic and Anti-Catabolic Therapy in Osteoporosis

Aims: Investigation of the mechanism by which CaMKK2 regulates osteoblast and osteoclast differentiation and devise potential strategies of its inhibition in the treatment of osteoporosis.

B. What other organizations were involved as partners?

Nothing to report.

8. Appendices

None.